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The Circular Bacteriocins Gassericin A and Circularin A

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Abstract: Gassericin A, a bacteriocin produced by *Lactobacillus gasseri* LA39, shows antibacterial activity against a number of Gram-positive food-borne pathogenic bacteria. Circularin A produced by *Clostridium beijerinckii* ATCC25752 is active against *C. tyrobutyricum*, a known cheese-spoilage bacterium. Both bacteriocins were purified to homogeneity from culture supernatants by reverse-phase chromatography and the subsequently determined amino acid sequences were used to clone the bacteriocin structural genes. Mature gassericin A and circularin A are class V circular bacteriocins comprised of 58 and 69 amino acid residues, respectively. Both bacteriocins are resistant to several peptidases and proteases, as are other cyclic bacteriocins. Heterologous expression of gassericin A in *Escherichia coli* was used to produce a non-cyclic mature peptide, which was shown to have a specific activity 173-fold lower than the circular molecule. The minimal region for production and secretion of active circularin A is comprised of five genes, as was deduced by heterologous gene expression in *Enterococcus faecalis*. Gassericin A and circularin A have limited mutual similarity in their primary sequences. Unlike most bacteriocins, including gassericin A, circularin A has a three-amino-acid-leader sequence.

INTRODUCTION

Bacteriocins are ribosomally synthesized anti-microbial peptides, proteins or proteinaceous complexes produced by bacteria that act mainly against closely related species. Wide-spectrum bacteriocins could play an important role in preventing the growth of harmful bacteria during the fermentation and preservation of various foods such as meats and dairy products [1, 2]. Bacteriocins from lactic acid bacteria (LAB) have been most widely studied and have been grouped into four classes I-IV [3, 4]. Lantibiotics are peptides containing modified amino acid residues such as lanthionine, β -methyl lanthionine, and dehydrated residues and belong to class I. Large proteins are part of class III, while the least studied group (class IV), contains complex molecules with lipids or carbohydrates. Class II contains small and heat-stable peptides and has been divided into the following categories: IIa, containing peptides with a consensus sequence (YGNGVXAAC) in the N-terminal of the mature bacteriocin; IIb, comprising peptides that require the complementary action of another peptide(s); IIc, which contains peptides that are secreted by the Sec-dependent pathway.

Recently, a new class, class V, has been proposed: that of the "cyclic bacteriocins" [5]. These peptides are characterized by the fact that their N- and C-termini are covalently linked. The first example of a cyclic bacteriocin was enterocin AS-48, produced by *Enterococcus faecalis* AS-48 (see review by Maqueda *et al.*, in this volume). Gassericin A, produced by *Lactobacillus gasseri* LA39 and

circularin A, produced by *Clostridium beijerinckii* ATCC25752, are further examples of this group of anti-microbial peptides and will be discussed in this paper.

Strains of the *Lactobacillus acidophilus* group of homofermentative LAB [6-8] are found in human and animal intestines and are added as dietary adjuncts to commercial fermented milk products, such as acidophilus yogurt, because of their physiological functions as "probiotics": they can survive and grow in the gastrointestinal tract [9]. The intake of these bacteria may have beneficial effects on human and animal health [9, 10]. There are a number of reports on the production of bacteriocins by strains of the six subgroups of *L. acidophilus*. *L. johnsonii* VPI11088 (lactacin F) [11, 12], *L. amylovorus* LMG P-13139 (lactobin A) [13], *L. amylovorus* DCE471 (amylovorin L471) [14] and *L. gasseri* SBT2055 (gassericin T) [15] produce similar bacteriocins. Lantibiotics have never been detected in strains of *L. acidophilus*.

In contrast to LAB, which are used for food and feed production, many species of clostridia are known as food-spoilage bacteria [16], and as pathogens and toxin producers [17]. Various clostridia produce bacteriocins such as BCN5 (*Clostridium perfringens*) [18] and boticin B (*C. botulinum*) [19]. Although bacteriocin production has been used in clostridia strain typing [20,21], there have been only a few studies on the molecular aspects of the regulation of bacteriocin production and secretion by clostridia, let alone on structure and function relationships.

In what follows we will review the literature on two circular bacteriocins identified in two functionally differing bacterial species, one a food-producer (*L. gasseri*), the other a food-spoiler (*C. beijerinckii*), gassericin A and circularin A, respectively.

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SELECTION AND PURIFICATION OF GASSERICIN A, A BACTERIOCIN PRODUCED BY *L. GASSERI*

From 70 bacteriocin-producing *Lactobacillus* strains isolated from human fecal specimens, the most widely effective bacteriocin was gassericin A produced by *L. gasseri* strain LA39 [22, 23]. Gassericin A showed antibacterial activity against Gram-positive food-borne pathogenic bacteria such as *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus*, but had no inhibitory activity against Gram-negative microorganisms such as *Salmonella typhimurium*, *S. enteritidis*, and *Escherichia coli* [24]. Gassericin A was purified to homogeneity from the supernatant of a culture grown in a modified MRS broth by reverse-phase chromatography [25, 26] and shown to have a molecular weight of 5,652 by mass spectrometry. This molecular weight was different from that estimated by SDS-PAGE (3,800). The purified bacteriocin was highly hydrophobic and did not dissolve in water.

SELECTION AND PURIFICATION OF CIRCULARIN A, A BACTERIOCIN PRODUCED BY *C. BEIJERINCKII*

From 12 clostridial strains tested, circularin A produced by *C. beijerinckii* ATCC25752 was the most effective bacteriocin against *C. tyrobutyricum* NIZOB570, a known cheese-spoilage bacterium [5]. Circularin A was purified to homogeneity from the supernatant of a culture grown in an AC broth dialysate by reverse-phase HPLC and was shown to have an apparent molecular weight of 2,200 by SDS-PAGE. Circularin A showed antibacterial activity against *C. tyrobutyricum*, *E. faecalis*, *L. alimentarius*, *L. sake* and *Lactococcus lactis* [5].

GASSERICIN A: STRUCTURAL GENE AND AMINO ACID SEQUENCE

After digestion of purified gassericin A with lysylendopeptidase and subsequent determination of the N-terminal amino acid sequence of the digestion product, the structural gene of gassericin A was cloned by reverse genetics. The gene, *gaaA* (DDBJ, EMBL, and GenBank databases, accession number AB007043), was shown to encode a protein of 91 amino acid residues with a calculated molecular weight of 9,286 [26].

Purified gassericin A was digested by lysylendopeptidase and 3-bromo-3-methyl-2-(2-nitrophenyl-mercapto)-3H-indole, which cleaves the tryptophanyl bonds, and the liberated peptides were sequenced by N-terminal protein sequencing [27]. All amino acid residues of gassericin A were identified in this way and the derived amino acid sequence was the same as that deduced from the structural gene *gaaA*. The molecular weight, 5,652 by mass-spectrometry, of gassericin A conformed to the weight expected after the elimination of H₂O from the deduced amino acid sequence. Mature gassericin A is a cyclic (class V) bacteriocin of 58 amino acid residues, in which the N-(Ile) and C-(Ala) terminal ends are covalently linked [27].

In silico analysis revealed that only two bacteriocins showed considerable sequence similarity to gassericin A (Fig. 1). One of these, acidocin B produced by *L. acidophilus* M46, has 98% identity with the gassericin A precursor but was not identified as a circular bacteriocin [28] (GenBank accession number Z34920). The mature bacteriocins differ in only one amino acid residue. Acidocin B has an apparent molecular weight of 2,400 as measured by SDS-PAGE, while the deduced molecular weight is 5,750. The other molecule is butyriovibriocin AR10 produced by the ruminal anaerobe *Butyriovibrio fibrisolvens* AR10 [29,30]. The cyclic bacteriocin (GenBank accession number AF076529) has 45% identity with gassericin A. N- and C-terminal regions of mature gassericin A before circularization have especially high similarities with those predicted for butyriovibriocin AR10. This would indicate that these regions are involved in processing and cleavage to form the circular bacteriocins or are important for bacteriocin activity.

CIRCULARIN A: STRUCTURAL GENE AND AMINO ACID SEQUENCE

After cleavage of purified circularin A using cyanogen bromide, which cleaves methionyl bonds, and subsequent determination of the N-terminal amino acid sequence of the cleavage products, the structural gene of circularin A was cloned by reverse genetics [5]. The gene, *cirA* (GenBank databases, accession number AY164463), was shown to encode a protein of 72 amino acid residues. From the internal amino acid sequences of peptide fragments of circularin A and the amino acid sequence deduced from *cirA*, circularin A was concluded to be a cyclic bacteriocin (class

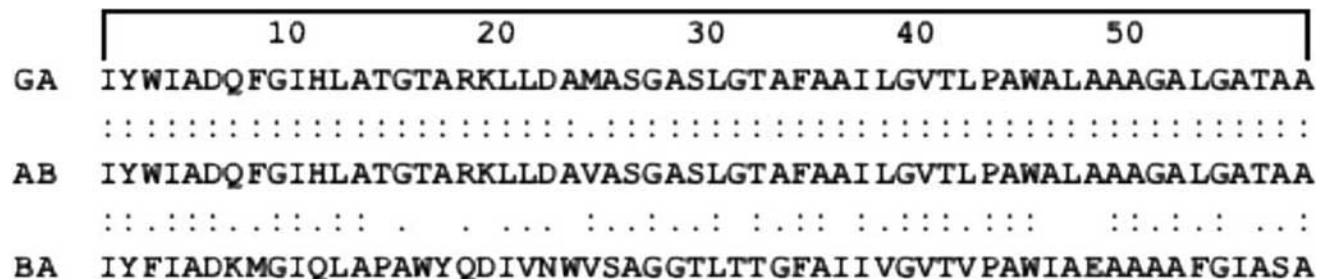


Fig. (1). Comparison of the primary amino acid sequences of gassericin A (GA, mature), acidocin B (AB, putative) and butyriovibriocin AR10 (BA, putative). Colons and periods show identical and homologous amino acids, respectively. The overlining indicates the circularization point.

	10	20	30	40	50	60
CirA	VAGALGVQTAAATTIVNVILNAGTLVTVLGI	IASIASGGAGTLMTIGWATFKATVQKLAKQSMARAIAY				
As48	MAKEFGIPAAVAGTVLNVVEAGGWVTTIVS	ILTAVGSGGLSLAAAGRESIKAYLKKEIKKKGKRAVIAW				

Fig. (2). Comparison of the primary amino acid sequences of circularin A (CirA) and enterocin AS-48 (AS48). Identical and homologous amino acids are indicated by colons and periods, respectively. Underlined amino acids: positions of the α -helices in the crystal structure of bacteriocin AS-48 as measured in N-decyl-beta-D-maltoside [50]. The over and underlining show the circularization of the molecules.

V) that is covalently closed at N-(Val) and C-(Tyr) terminal amino acid residues. Mature circularin A comprises 69 amino acid residues. A BLAST search revealed that circularin A has 60% similarity (31% identity) with the cyclic bacteriocin enterocin AS-48, which contains 70 amino acids (Fig. 2). In contrast to the situation in gassericin A and butyrivibriocin AR10, enterocin AS-48 and circularin A have only limited mutual similarity.

HETEROLOGOUS EXPRESSION OF GASSERICIN A AND CIRCULARIN A

Mature non-cyclic gassericin A was fused with a protein tag and expressed in *E. coli*. The specific activity of purified mature gassericin A, liberated from the fusion protein through digestion with factor Xa, was deduced to be 173-fold lower than that of purified native mature gassericin A [31].

The minimal region for production and secretion of active circularin A has been identified by heterologous expression of various deletion variants of the *cir* operon in *E. faecalis*. In this way, five genes were identified as minimally required [32]. This strategy also pinpointed the genes involved in circularin A immunity, namely *cirE*, the true immunity gene, and the combination of *cirB* and *cirD*, which together give additional resistance.

Secretion of LAB bacteriocins has been shown to proceed in either of three ways: some bacteriocins are

secreted via the general *sec*-pathway of protein secretion, the LsbA and LsbB bacteriocins of *Lc. lactis* are externalized via multidrug resistance (MDR) proteins [33], while still other bacteriocins are secreted via dedicated ABC-type transporters, for which the genetic information is located in the direct vicinity of the bacteriocin structural genes. Many class II bacteriocins are released after being cleaved at a conserved Gly-Gly motif at positions -2 and -1 in the processing site by a proteolytically active domain in the C-terminus of the dedicated secretion protein [34,35]. Although their leader peptide sequences show similarities, the sequences of the mature bacteriocins differ widely. ABC-type transporter genes are found in the direct vicinity of the structural genes of all known and putative circular bacteriocins, suggesting that dedicated transporters are involved in the secretion of these proteins, although none of these transporters have been proven to be involved in bacteriocin secretion as yet.

The putative leader peptide sequence of gassericin A contains 33 amino acid residues and does not contain the Gly-Gly motif. It is more similar to the leader sequence of nisin (Fig. 3). The processing site of gassericin A, predicted by the program SignalP V2.0 (<http://www.cbs.dtu.dk/services/SignalP/>), was the same as the actual site in gassericin A. A gene encoding a protein with a putative ATP binding domain is located downstream of *gaaA*, suggesting that it may be involved in the secretion of gassericin A (data not published).

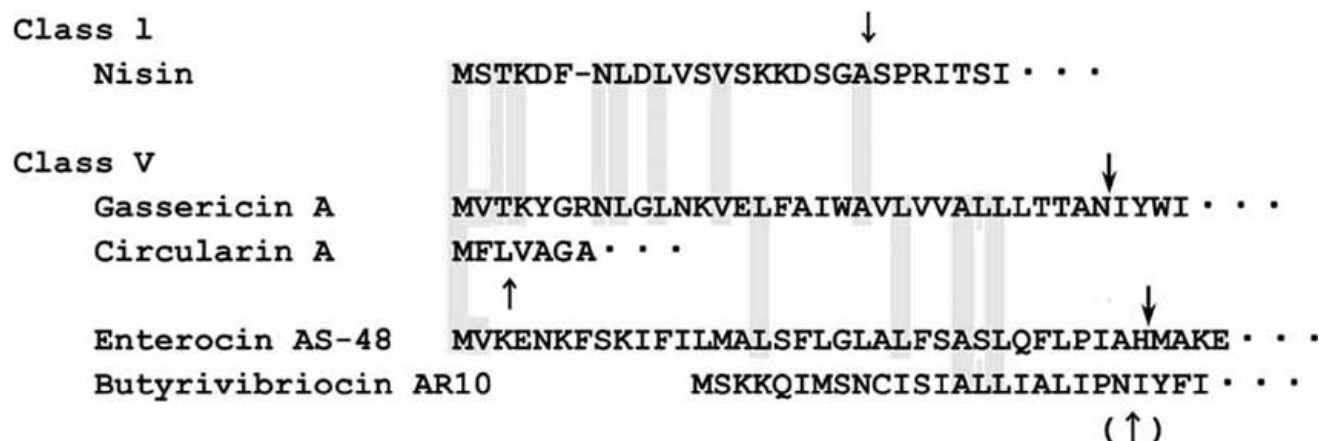


Fig. (3). Comparison of the leader peptide sequences of gassericin A and circularin A with those of nisin, enterocin AS-48 and butyrivibriocin AR10 (putative). The arrows show processing sites in mature bacteriocins (putative site in parenthesis). The hyphen was added to give optimum alignment.

In contrast, only 3 amino acids are cleaved from the circularin A precursor to obtain an active, secreted circular molecule [5]. This is quite different from enterocin AS-48, in which the processing site is similar to that of gassericin A and the putative site of butyrylvibriocin AR10 (Fig. 3).

LIMITED PROTEOLYSIS OF CYCLIC BACTERIOICINS

Gassericin A (Fig. 4) and circularin A are quite resistant to several peptidases and proteases [5,31], as are the other cyclic bacteriocins, microcin J25 [36] and enterocin AS-48 [37]. The resistance of the latter bacteriocin might be due to the compact globular structure of the molecule (see review by Maqueda *et al.*, in this volume) and it could explain the proteolytic stability of the other circular bacteriocins, if they too have similar compact structures. In fact, non-cyclic gassericin A purified from *E. coli* and cyclic gassericin A both migrated as peptides with a molecular weight of 3,800 in SDS-PAGE despite the actual molecular weights of 5666 and 5,652, respectively. This discrepancy may be due to the tertiary structure of the peptide.

REUTERICIN 6, A CYCLIC BACTERIOICIN IDENTICAL TO GASSERICIN A

L. reuteri is a gas-forming, heterofermentative LAB [38] and some strains are known antibiotic producers (e.g. of reuterin [39] and reutericyclin [40]). In our laboratory of Animal Products Chemistry, Toba *et al.* [41] detected reuterin 6, a narrow-spectrum bacteriocin produced by *L. reuteri* LA6 that also inhibits the growth of *B. cereus* [24]. Kabuki *et al.* [42] isolated reuterin 6 from Tween 80-free MRS diffusate broth, as the molecule, like gassericin A [25], showed strong affinity with Tween 80. Reuterin 6 was purified to homogeneity and was shown by amino acid sequence analysis to be a cyclic bacteriocin identical to gassericin A [43]. Nevertheless, the activity spectra of partly purified reuterin 6 and gassericin A do seem to differ [24,41]. The bacteriocin structural gene is located on the chromosome of *L. reuteri* LA6. Although this is the first example of identical bacteriocins in strains of the *L. acidophilus* group of LAB, identical bacteriocins have been reported in different other LAB species such as pediocin AcH in *Pediococcus acidilactici* and *L. plantarum* [44, 45]

and curvacin A-sakacin A in *L. curvatus* and *L. sake* [46,47]. It is highly possible, but impossible to prove, that the genes were transferred between *L. reuteri* and *L. gasseri*, as both strains were isolated within a 2-months interval from the feces of the same human infant.

CONCLUDING REMARKS

The circular bacteriocins gassericin A and circularin A have been purified, their amino acid sequences chemically determined and the bacteriocin structural genes have been cloned. Although we have started to learn about the genetics involved in gassericin A and circularin A production, a lot still needs to be examined:

1. Nothing is known about the mode of action of the two bacteriocins.
2. The genes involved in circularin A immunity have been identified [32]. We are currently examining which factors are involved in determining the resistance to gassericin A.
3. The genes and proteins involved in bacteriocin secretion are unknown for either bacteriocin. What seems to be clear is that neither is secreted via a Gly-Gly motif dedicated secretory system. Pre-gassericin A has an extended N-terminal leader that may be involved in the secretion process. Circularin A is singular in the fact that only 3 amino acid residues are cleaved off from the pre-bacteriocin to form the active, secreted peptide. Whether this cleavage is needed for secretion is not known at the moment.
4. It could be that the information for circularization of circularin A is, in fact, encoded within this 3-amino-acid leader. The process of circularization, which signals in the (pre-) bacteriocin are involved, which enzyme(s) performs the closing of the molecule, the site at which this process takes place and whether or not it is directly coupled to the secretion process, are all questions that need to be answered. The fact that we have the nucleotide sequences of the structural genes and the surrounding regions of *gaaA* and *cirA* and the possibility of doing heterologous gene expression studies opens up avenues to tackle these problems in the near future.

theoretical site

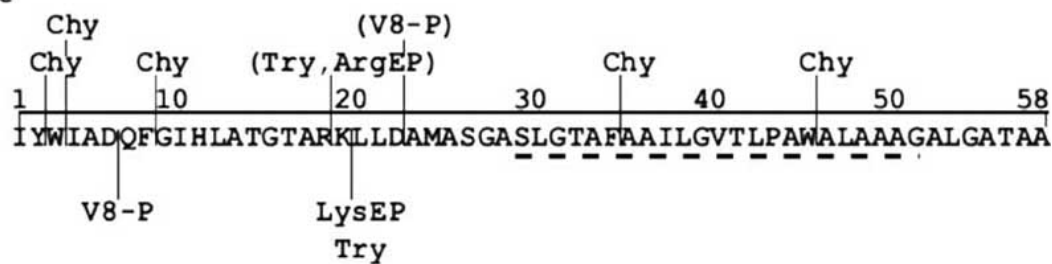


Fig. (4). A map of protease and peptidase cleavage sites in gassericin A. Theoretical cleavage sites are shown above the amino acid sequence, actual cleavage sites (confirmed by N-terminal amino acid sequencing) are presented below the sequence. Between brackets: upon incubation with the indicated enzyme gassericin A was found not to be cleaved. Chy: chymotrypsin, ArgEP: arginylendopeptidase, LysEP: lysylendopeptidase, Try: trypsin, and V8-P: V8 protease cleavage sites. The dotted line shows the putative α -helical membrane-spanning domain that could act on the cytoplasmatic membrane of sensitive cells. Overlining: circularization point.

5. Another point that needs to be addressed is the 3D-structure of the two bacteriocins. Enterocin AS-48 and circularin A have similar hydrophobicity profiles, similar isoelectric points, similar distribution of glycine residues and putative secondary structures, in addition to (limited) homology of primary sequences. This would indicate that the 3D-structure of circularin A might resemble that of enterocin AS-48. The determination of the 3D-structure of gassericin A has met with the problem that the purified bacteriocin does not dissolve in water.

Although the primary sequences of gassericin A and reuterin 6 are the same, the partly purified bacteriocins appear to have different anti-microbial spectra. Although we (Y. K. and T. S.) cannot at this stage exclude the presence of other anti-microbial components in the bacteriocin preparations, we are also examining the possibility that the difference is caused by distinguished post-translational modification mechanisms in the two producers (eg. D-/L-amino acid conversions) [48].

Circularin A is produced by *C. beijerinckii* and works effectively against clostridial strains. Although the mode of action and safety of circularin A remain to be examined, as well as the performance of the bacteriocin in food matrices, this anti-microbial peptide could possibly be used to prevent food spoilage by Clostridia. As *L. gasseri* is present as a natural inhabitant of the human intestine, the bacteriocin and the producer strain, as a probiotic, may be used safely and to the benefit of human health [49]. Many strains of *L. gasseri* only grow in expensive synthetic broths while only a few strains grow in milk-based media. The development of natural and low-cost broths for growth of *L. gasseri* may in the near future provide opportunities for more varied applications of the gassericin A producer, such as in treatment of bovine mastitis and in fermented milk products for human consumption.

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